TÜBİTAK

Turk J Biol
34 (2010) 379-387
© TÜBİTAK
doi:10.3906/biy-0901-17

Biological activities of Cu(II) and Hg(II) complexes of a heptadentate Schiff base ligand

Emel ERGENE[1], Hülya SİVAS[1,*], Kadriye BENKLİ[2]

[1]Department of Biology, Faculty of Science, Anadolu University, 26470 Eskişehir - TURKEY

[2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Anadolu University, 26470 Eskişehir - TURKEY

Received: 15.01.2009

Abstract: Two metal complexes were synthesized as [Hg**(L)**]$(ClO_4)_2$ and [Cu**(L)**]$(ClO_4)_2$ by the template reaction between 2,6-bis(2-aminothiophenoxymethyl)pyridine and 2,2'-bipyridine-6,6'-dicarboxaldehyde in the presence of Hg(II) and Cu(II) perchlorate salts. The structures of the compounds were elucidated by IR, ^{1}H-NMR, MASS, and Elemental Analyses values. Cytotoxicity of the compounds were investigated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay in normal and cancerous rat fibroblasts. Both compounds showed cytotoxicity on 2 cell lines as a dose-dependent manner. Compound [Hg**(L)**]$(ClO_4)_2$ was more cytotoxic than [Cu**(L)**]$(ClO_4)_2$. Apoptotic activity of compounds was evaluated by acridine orange staining and DNA fragmentation assay. Both cell lines exposed to the compounds exhibited condensed chromatin and appearance of apoptotic bodies. The percentages of all these abnormalities were found to be very high level in *ras*-transformed 5RP7 fibroblasts. The effect of [Hg(L)]$(ClO_4)_2$ was again more stronger than the compound [Cu(L)]$(ClO_4)_2$. Although the compound [Cu(L)]$(ClO_4)_2$ induced the formation of DNA fragmentation in both cell lines, [Hg(L)]$(ClO_4)_2$ induced the DNA fragmentation only in cancerous 5RP7 cells, indicating specific activity. In conclusion, we suggest that both complexes, especially the one with Hg(II) according to its significant differences of apoptotic morphology and DNA fragmentation, exhibit promising potentials as anticancer compounds.

Key words: Metal complexes, apoptosis, DNA fragmentation, cancer cells, MTT

Bir heptadentat Schiff baz ligandının Cu(II) ve Hg(II) kompleksinin biyolojik aktivitesi

Özet: İki metal bileşiği, [Hg**(L)**]$(ClO_4)_2$ ve [Cu**(L)**]$(ClO_4)_2$, Hg(II) ve Cu(II) perchlorate tuzlarının varlığında, 2,6-bis(2-aminothiophenoxymethyl)pyridine ve 2,2'-bipyridine-6,6'-dicarboxaldehyde arasındaki template reaksiyonu yoluyla sentezlenmiştir. Bileşiklerin yapısı IR, ^{1}H-NMR, MASS ve elemental analiz değerleri ile belirlenmiştir. Bileşiklerin sitotoksisitesi MTT [3-(4,5-dimetiltiyazol-2-yl)-2,5-difeniltetrazolyum bromid] yöntemiyle, normal ve kanserli sıçan fibroblastlarında araştırılmıştır. Her iki bileşik iki hücre hattı üzerine doza-bağımlı sitotoksıklik göstermiştir. [Hg**(L)**]$(ClO_4)_2$ bileşiği [Cu**(L)**]$(ClO_4)_2$'den daha fazla sitotoksiktir. Bileşiklerin apoptotik aktivitesi akridin turuncusu boyama ve DNA fragmentasyon yöntemleriyle değerlendirilmiştir. Bileşiklere maruz bırakılan her iki hücre hattı kromatin yoğunlaşması ve apoptotik kese oluşumunu göstermiştir. Tüm bu anomalilerin yüzdesinin *ras*-transform olmuş 5RP7 fibroblastlarında çok yüksek seviyede olduğu bulunmuştur. [Hg(L)]$(ClO_4)_2$ bileşiğinin etkisi [Cu(L)]$(ClO_4)_2$ bileşiğinden daha kuvvetli olmuştur. [Cu(L)]$(ClO_4)_2$ her iki hücre hattında da DNA fragmentasyonuna neden olurken [Hg(L)]$(ClO_4)_2$ bileşiği özgül bir aktivite göstererek yalnızca kanserli 5RP7 hücrelerinde DNA fragmentasyonuna neden olmuştur. Sonuçta, iki bileşiğin, özellikle apoptotik morfoloji ve DNA fragmentasyonu üzerine önemli derecede farklı etkisi bakımından Hg(II) ile olanın, anti-kanser potansiyele sahip olduğunu ileri sürmekteyiz.

Anahtar sözcükler: Metal bileşikleri, apoptoz, DNA fragmentasyonu, kanser hücreleri, MTT

Introduction

New strategies have been developed in synthesis of new therapeutic agents since most drugs used currently show many important side effects (1,2). Approval of cisplatin as a chemotherapeutic drug triggered an enormous response in the chemical and medical research communities for the design of new complexes by metal-ligand bonding derivatives (3-5). Therefore, a large number of findings, such as the decreased Cu-Zn SOD activity of tumor cells, the altered copper(II) metabolism in cancerogenesis (6), and breakage of DNA, have led to an explosive growth of interest in the synthesis of potential cytotoxic, genotoxic, anticarcinogenic, and apoptotic Cu(II) complexes (7-9). CopperII complexes of thiosemicarbazones were synthesized as potential antitumor agents and showed significant improvement in cytotoxic activity against human acute lymphoblastic leukemia CCRF-CEM cells and colon adenocarcinoma HT-29 cells (10).

Copper and mercury complexes, such as sulfadiazine complexes, are still used in topical medicine as an antiseptic, even though it is known that the interaction of heavy metal ions with biomolecules can have potentially toxic effects (11,12). Recently, synthesis and cytotoxic activities of some pyridine derivative compounds complexed with Cu(II) and Hg(II) against cancer cells have been reported (13-15).

In the present study, 2 metal complexes have been synthesized by the condensations of 2,6-bis(2-aminothiophenoxymethyl)pyridine and 2,2'-bipyridine-6,6'-dicarboxaldehyde with Hg(II) and Cu(II) perchlorate in methanol. The structures of the compounds were elucidated by IR, ^{1}H-NMR, MASS, and elemental analysis values. Cytotoxicity of these metal complexes was investigated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay in normal and cancerous rat fibroblasts. Cells were treated with a series of concentrations of test compounds for 24 and 48 h. Both compounds were found to be cytotoxic for 2 cell lines as a dose-dependent manner. $[Hg(L)](ClO_4)_2$ was more cytotoxic than $[Cu(L)](ClO_4)_2$. On the other hand, possible apoptotic activity of compounds was evaluated by DNA fragmentation assay and the results were discussed.

Materials and methods

Chemistry

Melting points were determined using a Gallenkamp MPD350.BM2.5 digital melting point apparatus and were uncorrected. The compounds were checked for purity by TLC on silica gel 60 F_{254} (Merck). Elemental analyses were performed on a CHNS-O Carlo Erba EA 1108 elemental analyzer; IR spectra were obtained with a Shimadzu 470 IR spectrophotometer using nujol mulls or KBr disc; ^{1}H NMR spectra were recorded with a Varian (300 MHz) or Bruker spectrometer (250 MHz) in $CDCl_3$ or DMF-d_7 as solvent. ^{13}C NMR spectra were recorded with a Varian (75.5 MHz) in $CDCl_3$ as solvent; MS-FAB$^+$ spectra were obtained with a Finnigan Mat 95 mass spectrometer.

General synthesis procedure

2,2'-bipyridine-6,6'-dicarboxaldehyde (0.18 g, 1 mmol) and the appropriate metal perchlorate salt (1 mmol) were dissolved in hot MeOH (25 mL) and 2,6-bis(2-aminothiophenoxymethyl)pyridine (0.36 g, 1 mmol) in methanol (25 mL) was added dropwise with stirring. The mixture were refluxed for 3-4 h and filtered hot. The solvent of the reaction mixture was reduced to half its original volume and then the mixture was placed in a refrigerator to induce crystallization. Desired product was filtered and dried (16).

$[HgL](ClO_4)_2$: Yellow solid, yield 64%. IR (KBr): ν(cm^{-1}) 1628 (C=N), 1587 (pyridine), 1094 and 624 (perchlorate anion). ^{1}H NMR (300 MHz, d_7-DMF): δ_H 4.36 (s, 4H, CH_2), 6.63-6.94 (m, 2H), 7.10-7.88 (m, 8H), 8.61-8.81 (m, 3H) 9.12 (d, J 7.0 Hz, 2H), 9.60 (s, 2H, HC=N). m/z: 691 $[CuL(ClO_4)]^+$, 592 $[CuL]^{2+}$.m/z: 830 $[HgL(ClO_4)]^+$, 731 $[HgL]^{2+}$. (Found: C, 39.1; H, 2.5; N, 7.1; S, 7.5. Calc. for $C_{31}H_{23}N_5S_2O_8Cl_2Hg.H_2O$: C, 39.3; H, 2.6; N, 7.4; S, 6.8)

$[CuL](ClO_4)_2$: Color of complex: brown, yield 35%; IR (KBr): ν(cm^{-1})1618 (C=N), 1587 (pyridine), 1091 and 625 (perchlorate anion). ^{1}H NMR (300 MHz, d_7-DMF): δ_H 4.34 (s, 4H, CH_2), 6.61-6.91 (m, 2H), 7.11-7.85 (m, 8H), 8.60-8.79 (m, 3H) 9.11 (d, J 7.1 Hz, 2H), 9.59 (s, 2H, HC=N). m/z: 691 $[CuL(ClO_4)]^+$, 592 $[CuL]^{2+}$.(Found: C, 46.6; H, 3.0; N, 8.5; S, 8.5. Calc. for $C_{31}H_{23}N_5S_2O_8Cl_2u.H_2O$: C, 46.9; H, 2.9; N, 8.8; S, 8.1).

Activity

Cell culture and treatments

F2408 (normal rat embryonic fibroblast) and 5RP7 (c-H-*ras* transformed-rat embryonic fibroblast) were donated by Institute for Fermentation, Osaka, Japan. Cells were grown in 10% Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco BRL), penicillin/streptomycin at 100 units/mL, and 2 mM L-glutamine, at 37 °C under 5% CO_2/95% air in a humidified atmosphere. An appropriate number of cells were seeded and incubated for 12 or 24 h prior to the treatment with a series concentration of test compounds and DMSO as a solvent control for 24 h and 48 h.

Preparation of test compounds

Test compounds to be tested were dissolved in absolute DMSO at a concentration of 5 mM as a main stock, and then further diluted to various concentrations using culture medium. Both compounds were tested at 3 to 5 concentrations.

Viability assay

Effects of the compounds on the viability of F2408 and 5RP7 cells were measured by the MTT assay. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay depends on the mitochondrial enzyme reduction of tetrazolium dye to determine cell viability. In the experiment, cells were plated at a density of 5×10^3 cells/well into 96-well plates and allowed to adhere for 24 h at 37 °C. Then the medium was replaced by 100 µL of culture medium supplemented with chemicals at specified concentrations for 24 and 48 h. At the end of the treatment, the culture medium in each well was replaced with 100 µL of culture medium containing MTT (Sigma) at a final concentration of 1mg/mL for additional 3 h incubation at 37 °C. The formazan grains formed by the viable cells were solubilized by the addition of 200 µL/well of DMSO. The absorbance was read on a Bio-Tek (ELX 808 IU) microplate reader at a wavelength of 540 nm. The signal generated is directly proportional to the number of viable cells in wells (17).

Detection of apoptosis by fluorescence microscopy

For the determination of apoptosis, F2408 and 5RP7 cells were seeded at a density of 2.5×10^5 cells/well into 6-well plates included cover slips. After 24 h adherence, the cells were treated with a serial dose of chemicals. Following 24 h incubation, cells were rinsed with cold PBS and fixed in ice-cold 70% ethanol for 5 min. After washing, the cells were stained with acridine orange (0.1 mg/mL) for 10 min and then rinsed with PBS. Cover slips were mounted on slides and changes in cell morphology were viewed and the abnormal cells were scored under a fluorescence photomicroscope (Olympus BX50).

DNA fragmentation evaluation

F2408 and 5RP7 cells were seeded at a density of 3×10^5 cells into the 25 cm flask, incubated for 24 h and then treated with compounds for further 24 and 48 h. Then DNA was extracted using the DNeasy Tissue Kit (QIAGEN Inc., USA) according to the manufacturer's instructions. Briefly, the cells were collected and then resuspended in 20 mL PBS. Following the addition of proteinase K and the lyses buffer, the sample was incubated at 70 °C for 10 min. After ethanol precipitation, DNA was collected from the sample by centrifugation at 8000 rpm for 1 min using a mini spin column. Then DNA was dried on a membrane and eluted by centrifugation after addition of an elution buffer. The presence of internucleosomal DNA cleavage was then analyzed using 1.5% agarose gel electrophoresis containing ethidium bromide, followed by observation under ultraviolet illumination.

Statistical analysis

Viability experiments were performed 3 times in triplicate and data were statistically analyzed with the One-way Anova test using SPSS.

Results and discussion

The complexes were synthesized by a template synthesis, in which the Schiff base macrocyclic ligand resulted from the condensation of 2,2'-bipyridine-6,6'-dicarboxaldehyde with 2,6-bis(2-aminothiophenoxymethyl)pyridine in the presence of Hg(II) and Cu(II) ions (Figure 1).

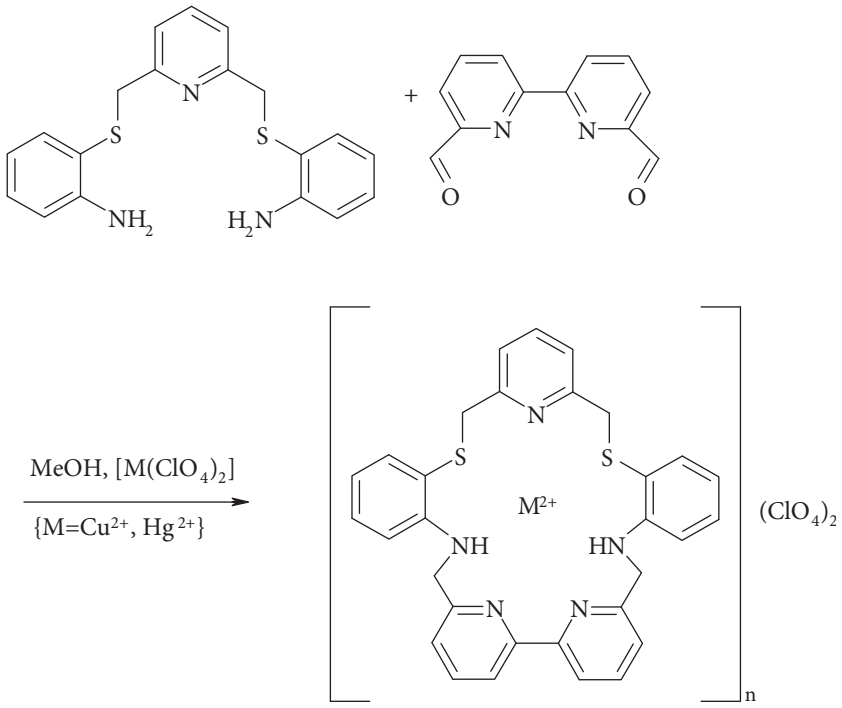

Figure 1. Synthetic route to the complexes.

The infrared spectra of all metal complexes in the region 400-4000 cm^{-1} show a strong absorption band around 1616-1638 cm^{-1}, which is assigned to the C = N stretching vibration, indicating the formation of the Schiff base products. For the metal complexes, absorptions at 1091-1094 and 624 cm^{-1} were assigned to the ν_3 and ν_4 stretching modes of ionic perchlorate (16). The peaks on ^{1}H-NMR spectra for the complexes were consisted with the given formulation. The composition of the complexes was also confirmed by elemental analyses and by fast atom bombardment mass spectrometry. At the mass spectrums of the complexes, 2 fragmentation patterns were observed corresponding to the sequential loss of the counter ion.

The in vitro cytotoxicity was evaluated after 24 and 48 h incubation of the synthesized compounds at increasing concentration with the normal and cancer cells. Results of the MTT assay are expressed in terms of OD values at 540 nm and presented in Figure 2. Both compounds exhibited cytotoxic activity at a very low concentration against any of 2 cell lines. However, $[Hg(L)](ClO_4)_2$ was more effective ($IC_{50} > 20$ μM) than the cytotoxicity of $[Cu(L)](ClO_4)_2$, because the IC_{50} values were less than 70 μM. Cytotoxic activity of $[Cu(L)](ClO_4)_2$ was slightly higher for cancerous 5RP7 cells when compared with the normal F2408 cells. On the other hand, $[Hg(L)](ClO_4)_2$ did not show any significant differences in the cytotoxic activity for 2 cell lines.

To determine whether the cytotoxic effect of the compounds was related to the induction of apoptosis, morphology of cells was investigated using the acridine orange staining. As shown in Figure 3, obvious morphological changes were observed in the treated cells as a dose-dependent manner compared to the solvent treated cells. Both cell lines exposed to the compounds exhibited condensed chromatin and appearance of apoptotic bodies as apoptotic indicators. Furthermore, the number of micronucleated and binucleated cells were significantly increased after the treatment with compounds (Table). Interestingly, the percentages of all these abnormalities were found to be very high level in *ras*-transformed 5RP7 fibroblasts as seen in Figure 4. Furthermore, the effect of $[Hg(L)](ClO_4)_2$ was found to be stronger than the compound $[Cu(L)](ClO_4)_2$ even at its lower concentrations. This was consistent with the results of the MTT assay.

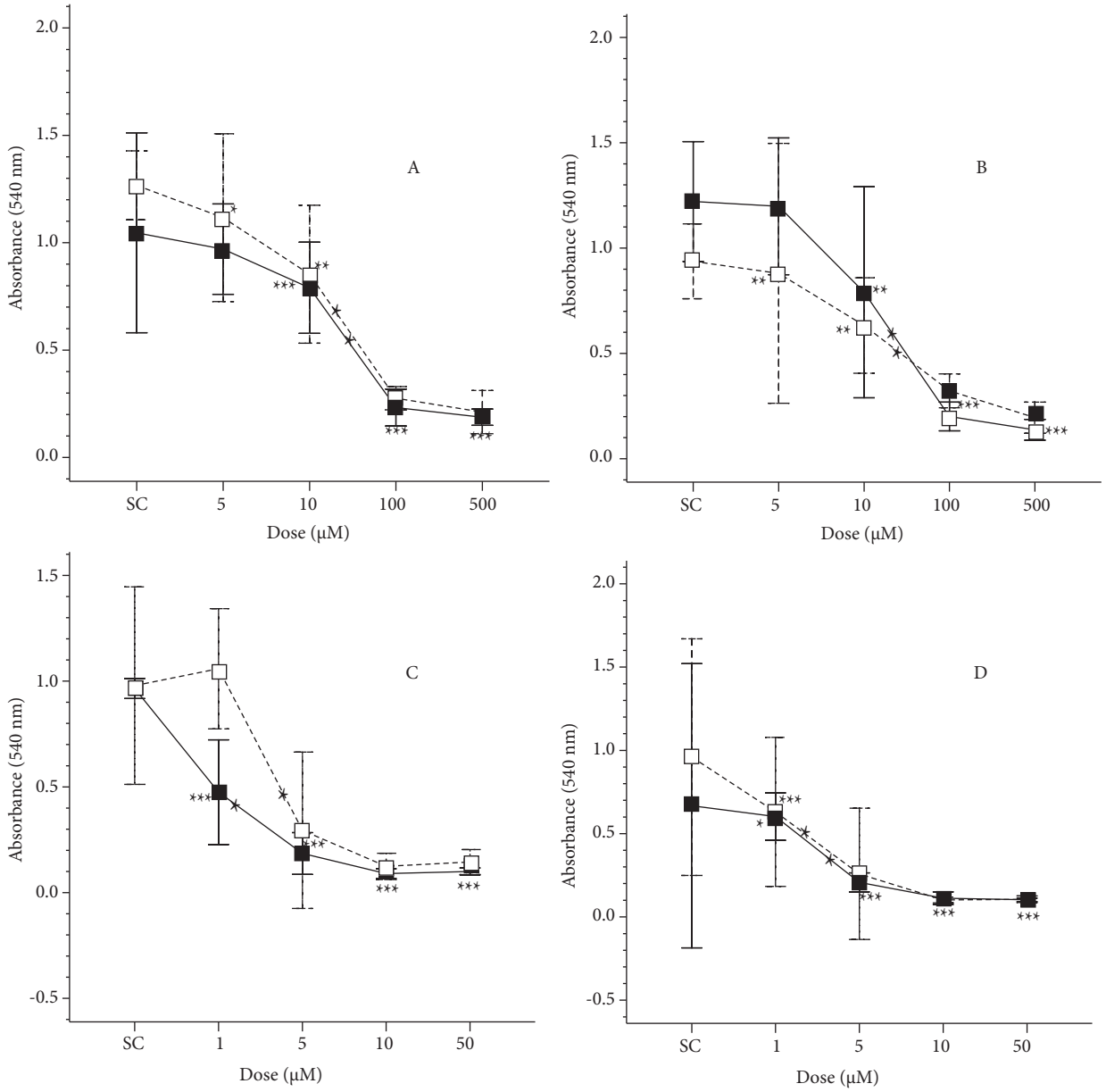

Figure 2. Effects of the chemicals on F2408 (A and C) and 5RP7 (B and D) cells viability using the MTT assay expressed as absorbance values at 540 nm. Cells were incubated for 24 or 48 h with $[Cu(\mathbf{L})](ClO_4)_2$ (A and B) and with $[Hg(\mathbf{L})](ClO_4)_2$ (C and D). Values are the mean ± S.E. of data from at least 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.005. *IC_{50}. —■— : 24 h, ···□··· : 48 h. SC: Solvent control.

The presence of cells with apoptotic bodies in cultured cells treated with cytotoxic agents were considered as a marker of cell death by apoptosis and linked to DNA fragmentation associated with apoptosis (18). Typical results from F2408 and 5RP7 cells treated with each of both compounds are shown in Figure 5. The compound $[Cu(L)](ClO_4)_2$ induced the formation of DNA fragmentation in the both cell lines compared to the control cells (Figure 5). However, the compound $[Hg(L)](ClO_4)_2$ induced the DNA fragmentation only in cancerous 5RP7 cells, not in the normal F2408 cells, indicating a very excited specific activity. Results of DNA fragmentation were also consistent with the results of apoptotic cell

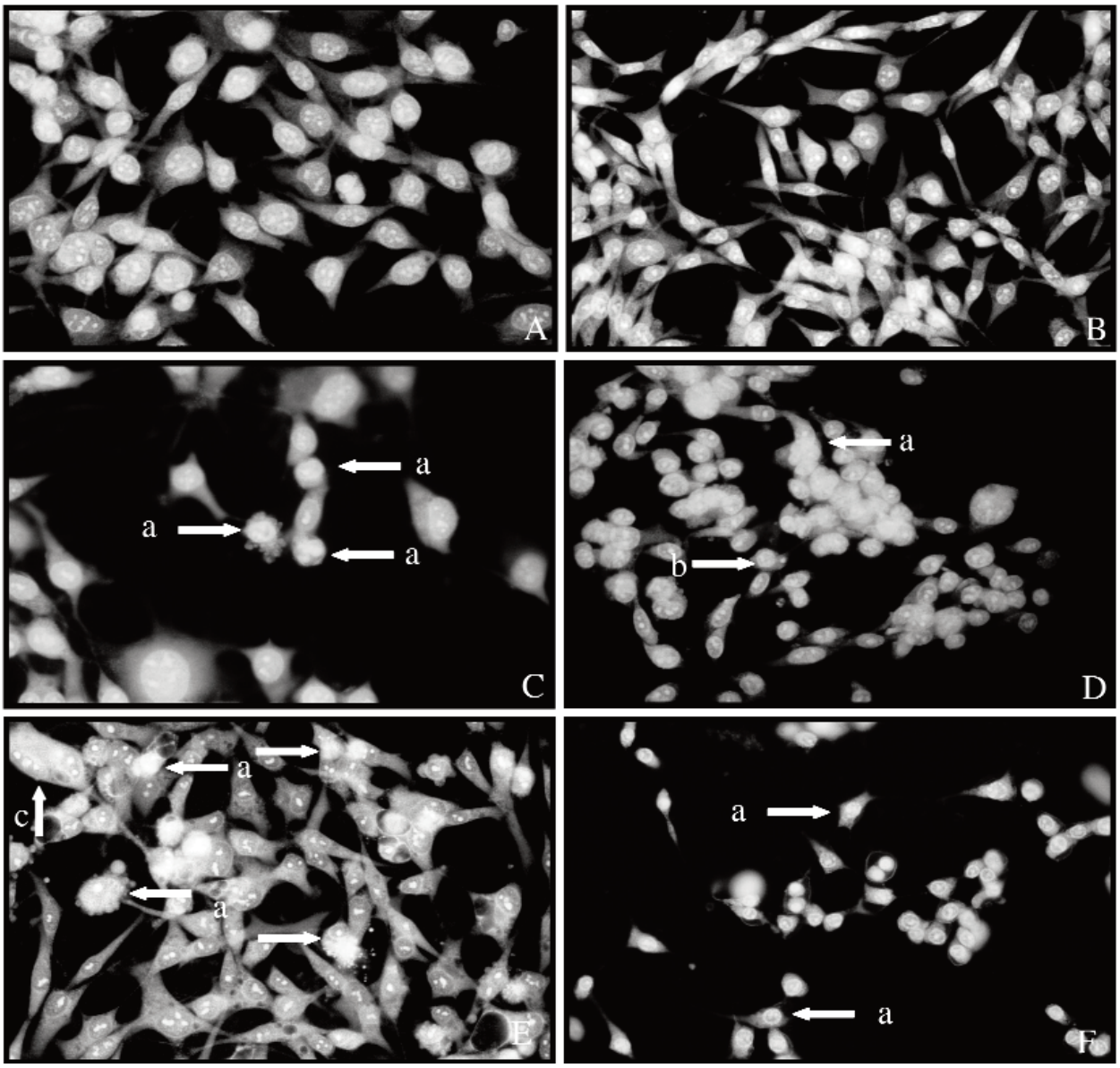

Figure 3. Morphology of F2408 (A, C, E) and 5RP7 (B, D, F) cells was visualized under a fluorescence microscope after acridine orange staining. DMSO treated F2408 (A) and 5RP7 (B) cells as controls, F2408 (C, E) and 5RP7 (D, F) cells after 24 h treatment with 1 μM or 5 μM of [Hg(**L**)]$(ClO_4)_2$. Apoptotic cells with cytoplasmic and nuclear blebs and condensed chromatin (a), micronucleated cells (b) and binucleated cells (c). Mag: 400×.

morphology. In untreated or DMSO treated cells, there was no DNA fragmentation, and intact genomic DNA was situated near the well at the top of the lanes.

Recently, Zhao et al. (13) reported similar results to our findings indicating the cytotoxic effects of Cu(II) and Hg(II) complexes with N-[2-}(pyridine-2-ylmethylidene)amino}ethyl]acetamide on neoplastic cells. However, they found that Cu(II) complex was more effective than Hg(II) complex of the compounds. Another Hg(II) complex of 2,6-bis(benzimidazol-2-yl) pyridine ligand showed broad-spectrum antimicrobial activity and bound effectively to calf thymus DNA (19). Furthermore, Cu(II) complexes of thiosemicarbazones were

Table. Abnormal cell number after treatment with the compounds for 24 h was scored using fluorescence staining assay.

Cell type: F2408		Total cell number	A	B	C	Total abnormal cell (%)
$[Cu(\mathbf{L})](ClO_4)_2$ (μM)						
	5	2000	44	9	8	3.0
	50	2650	172	4	20	7.3
	100	2550	175	26	30	9.0
$[Hg(L)](ClO_4)_2$						
	1	1500	10	1	3	0.9
	5	1500	24	-	16	2.6
	10	1500	108	29	17	10.2
DMSO (v/v)	0.1%	1500	7	3	8	**1.2**
Cell type: 5RP7						
$[Cu(\mathbf{L})](ClO_4)_2$	5	1650	235	18	17	16.3
	50	1120	195	3	27	20.0
	100	270	224	10	15	92.2
$[Hg(L)](ClO_4)_2$						
	1	1200	298	42	19	29.9
	5	1500	982	3	6	66.0
	10	500	232	8	17	51.4
DMSO (v/v)	0.1%	1500	29	2	13	**2.9**

DMSO: dimethylsulfoxide as a solvent control, A: Apoptotic cells, B: Cells with micronucleus, C: Binucleated cells.

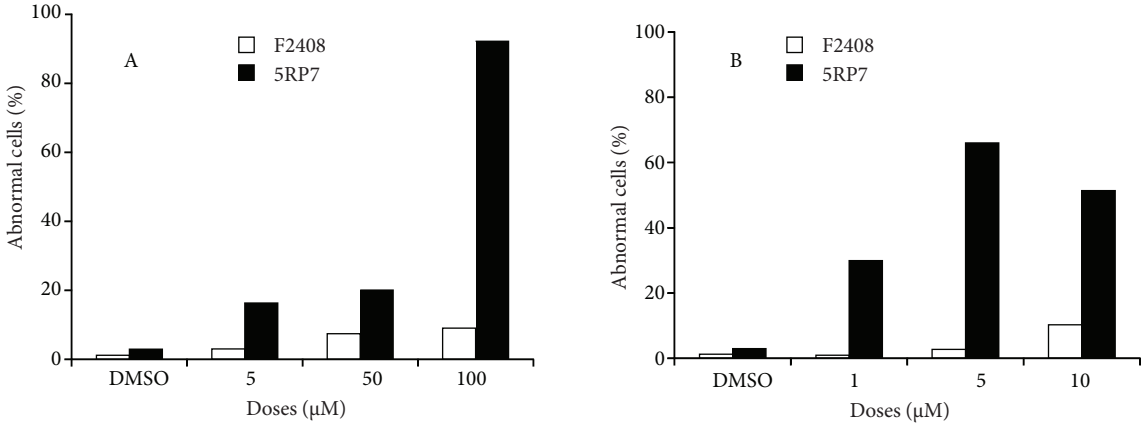

Figure 4. Percentages of the abnormal F2408 and 5RP7 cells treated with $[Hg(\mathbf{L})](ClO_4)_2$ (A) and $[Cu(\mathbf{L})](ClO_4)_2$ (B) presented in Table 2 are expressed as graphs.

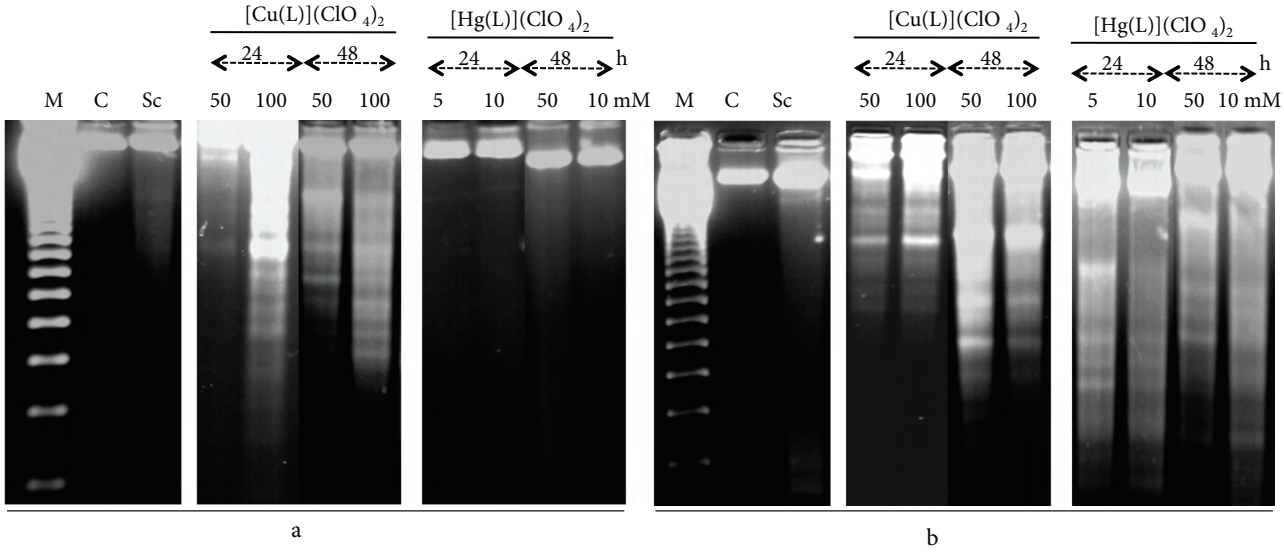

Figure 5. DNA fragmentation in F2408 (a) and 5RP7 (b) cells by agarose gel electrophoresis after growth in the presence of [Cu(**L**)](ClO$_4$)$_2$ and [Hg(**L**)](ClO$_4$)$_2$ for 24 and 48 h. M: ladder marker, C: untreated control cells, Sc: in the presence of DMSO as solvent control. A representative result from at least 3 independent experiments is shown.

reported to show significant improvement in cytotoxic activity against human acute lymphoblastic leukemia CCRF-CEM cells and colon adenocarcinoma HT-29 cells. These compounds are also noncell cycle specific agents and they were found to be potent inducers of apoptosis in Burkitt's lymphoma cells (10). Here, appearances of apoptotic morphology, DNA fragmentation, and the formation of micronucleus may be a result of the binding of compounds to DNA.

In conclusion, the present results obtained using the 2 rat cell lines, show that both complexes, especially the one with Hg(II), exhibit promising potentials as an anticancer compounds according to significant differences of apoptotic morphology and DNA fragmentation.

Corresponding author:

Hülya SİVAS,

Department of Biology,

Faculty of Science,

University of Anadolu,

26470 Eskisehir - TURKEY

E-mail: hzeytino@anadolu.edu.tr

References

1. Mothilal KK, Karunakaran C, Rajendran A et al. Synthesis, X-ray crystal structure, antimicrobial activity and photodynamic effects of some thiabendazole complexes. J Inorg Biochem 98: 322-332, 2004.
2. Agh-Atabay NM, Dulger B, Gucin F. Structural characterization and antimicrobial activity of 1,3-bis(2-benzimidazyl)-2-thiapropane ligand and its Pd(II) and Zn(II) halide complexes. Eur J Med Chem 40: 1096-1102, 2005.
3. Pectasides D, Kamposioras K, Papaxoinis G et al. Chemotherapy for recurrent cervical cancer. Cancer Treat Rev 34: 603-613, 2008.
4. Rosenberg B, VanCamp L, Trosko JE et al. Platinum compounds: A new class of potent antitumour agents. Nature 222: 385-386, 1969.
5. Pasetto LM, D'Andrea MR, Brandes AA et al. The development of platinum compounds and their possible combination. Crit Rev Oncol Hematol 60: 59-75, 2006.
6. Sorenson JRJ. Copper complexes offer a physiological approach to treatment of chronic diseases. Prog Med Chem 26: 437-568, 1989.

7. Raptopoulou CP, Paschalidou S, Pantazaki AA et al. Bis(acetate)bis(1-methyl-4,5-diphenylimidazole)copper(II): Preparation, characterization, crystal structure, DNA strand breakage and cytogenetic effect. J Inorg Chem 71: 15-27, 1998.

8. Beynek N, Uluçam G, Ayaz Tuylu B et al. Synthesis, characterization of a new macro cyclic legend and its copper(II), cadmium(II) and lead (II) complexes: Genotoxic activity of these complexes in cultured human lymphocytes. Drug Chem Toxicol 30: 399-410, 2007.

9. Chen SH, Lin JK, Liang YC et al. Involvement of activating transcription factors JNK, NF-kB, and AP-1 in apoptosis induced by pyrrolidine dithiocarbamate/Cu complex. Eur J Pharmacol doi: 10.1016/j.ejphar. 2008.07.024.

10. Easmon J, Pürstinger G, Heinisch G et al. Synthesis, Cytotoxicity, and Antitumor Activity of Copper(II) and Iron(II) Complexes of 4N-Azabicyclo[3.2.2]nonane Thiosemicarbazones Derived from Acyl Diazines. J Med Chem 44: 2164-2171, 2001.

11. Anand N. Sulfonamides and sulfanos. In: Wolff ME, ed. Burger's Medicinal Chemistry. Wiley Interscience, New York; 1980: pp. 1-40.

12. Constable EC. Complexes and aqueous solution chem. of Hg(II), complexes of Hg(I), organomercury(II) complexes, compounds with Hg-heterometal bonds, and Hg in organic synthesis. Coord Chem Rev 62: 37-83, 1985.

13. Zhao X, Peng-Foo Lee P, Yan Y–K et al. Synthesis, crystal sutructures and cytotoxicities of some transition metal complexes with N-[2-{(pyridine-2-ylmethylidene)amino} ethyl]acetamide. J Inorg Biochem 101: 321-328, 2007.

14. Shrivastav A, Singh NK, Tripathi P et al. Copper(II) and manganese(III) complexes of N'-[(2-hydroxy phenyl)carbonothioyl] pyridine-2-carbohydrazine: novel therapeutic agents for cancer. Biochimie 88: 1209-1216, 2006.

15. Gokhale N, Padhye S, Rathbone D et al. The crystal structure of first copper(II) complex of a pyridine-2-carboxamidrazone – a potential antitumor agent. Inorg Chem Comm 4: 26-29, 2001.

16. Uluçam G, Beynek N, Seller Z et al. Synthesis, characterization of some transition metal complexes of a new heptadentate N5S2 schiff base ligand and the effects of these metal complexes on U2OS cells cytotoxicity and DNA cleavage activity. Phosp Sulf Sil Rel Elem 183: 2237-2247, 2008.

17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63,1983.

18. Evan G, Littlewood T. A Matter of life and cell death. Science 281: 1317-1322, 1998.

19. Agh-Atabay NM, Neshat A, Karabiyik T et al. Synthesis, characterization and antimicrobial activity of Fe(II), Zn(II), Cd(II) and Hg(II) complexes with 2,6-bis(benzimidazol-2-yl) pyridine ligand. Eur J Med Chem 42: 205-213, 2007.